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Arsenite As(III) Stress on *Arabidopsis thaliana* MRP1 AtABCC1 Plant Growth

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Abstract

ABC transporters are membrane bound proteins involved in the transport of a broad range of amphipathic molecules across membranes. The superfamily of ABC transporters contain highly represented subfamilies and MRPs (ABCCs) are one of them. Plant MRPs like *Arabidopsis thaliana* MRP1 also transport various glutathione conjugates and heavy metals like arsenic across membranes and is already known to be involved in vacuolar transport of folates. In presence of increasing amount of arsenite (AsIII) salt the plants show less root and shoot growth in *Arabidopsis thaliana* MRP1 T-DNA homozygous mutants compared to wild type plants. Results showed that wild type AtMRP1 is tolerant against arsenite stress because of AtMRP1 function and earlier results showed MRP1 phosphorylation is a potential regulator in this tolerance. Here phospho-serine antibody cross reacted with *Arabidopsis thaliana* wild type plant leaf extracts only in absence of arsenite which indicates the change in protein phosphorylation and expression in presence of arsenite. To understand the effects of arsenite on AtMRP1 wild type and mutant plant growth experiments were done. The experiments provided an important insight on AtMRP1 plants under arsenite stress conditions.

INTRODUCTION

ATP Binding Cassette (ABC) transporters are present in microorganisms, yeasts and mammals and are involved in the transport of chemically diverse compounds across membranes. Multidrug resistance protein (MRP/ ABCC) is a subfamily among the ABC superfamily of transporters shown to have important functions across evolution. In plants, *Arabidopsis thaliana* ABCC1 is present in vacuolar membranes and is known to transport glutathione (GSH) conjugate and folates [1,2]. Early studies with AtMRP1 showed MgATP-energized, vanadate-inhibitable, uncoupler-insensitive uptake of several glutathione (GSH) conjugates (GS-conjugates) [3,4]. In addition, AtMRP1 was shown to have a role in tolerance and detoxification of heavy metal and metalloids [1,5,6]. MRP proteins are classified based on the presence of one or two ATP-binding cassettes or nucleotide binding domains (NBDs). Each NBD encompasses ~200 amino acid residues and contains three idiotypic sequence motifs [7].

In addition to two NBDs, AtMRP transporters contain two or three hydrophobic integral membrane spanning domains (MSDs), each of which contains multiple (usually four to six) transmembrane α-helices. The MSDs form the pathway for solute movement across the phospholipid bilayer. AtMRP1 is localized at the vacuolar membrane and transports substrates to the vacuolar lumen thereby effectively sequestering them from cytosol [8,9].

In south Asia, particularly in parts of Indian subcontinent, arsenic in ground water is a hazardous environmental pollutant. Arsenic content in water used for drinking and irrigation has been reported to cross the accepted limits of 10 µg/L proposed by the World Health Organization in several areas of South Asia. Arsenic is taken up by plant roots and is sequestered in plant vacuoles. Plant vacuoles act as final detoxification stores for heavy metals and arsenic. Over expression of Ycf1 (35S::ycf1) in yeast was shown to confer both accumulation and enhanced resistance to lead and cadmium in *Arabidopsis* plants [10]. The essential transporters that sequester toxic metals in plant vacuoles have long been sought but remain unidentified in plants. Two transporters in *Arabidopsis*, from ABCC (MRP) family AtABCC1 and AtABCC2, were shown to contribute to arsenic tolerance via vacuolar sequestration of As (III)-phytochelatin conjugates. The most common forms of arsenic available in the environment for uptake by plants are arsenate (AsV) and arsenite (AsIII). Arsenate is acquired by plant roots through endogenous transport systems for phosphate and is reduced to the thiol-reactive form As III inside the cell [11]. At equimolar concentrations, AsIII is more toxic than AsV.

The experiments described here were directed towards understanding the role of arsenite effect in plant growth and phosphorylation events controlling AtMRP1 transporter function under AsIII stress treatment. There are several potential phosphorylation sites in AtMRP1 and using mutational analysis and heterologous expression in yeast, the role of phosphorylation, especially in the serine triad, has been investigated [6].

Experiments were done to understand the effects of arsenite on AtMRP1 plant growth. The data presented shows AtMRP1 plant growth and whether the plant NBD2 gets phosphorylated like the *in vitro* expressed proteins under AsIII stress condition. A potential signaling event in response to As III is also described in wild type and mutant plants.

MATERIALS AND METHODS

Chemicals

Hepes, glycerol, Triton X-100, β-mercaptoethanol, aprotinin, leupeptin, Protein A sepharose, PMSF and Western blotting kit were obtained from Sigma. Anti-GAPDH antibody was obtained from BioBharati Life Science (Kolkata, India) and Phospho-Serine antibody was obtained from Qiagen. Chromatography materials were obtained from GE lifesciences. All enzymes were obtained from New England Biolabs. All other chemicals and biochemicals were of analytical grade.

Plant materials and growth conditions

For the majority of the investigations of *Arabidopsis thaliana*, ecotype Wassilewskia (WS) was employed except for the analyses of atmrp1-2 knock-out mutants, which were performed on ecotype Columbia (Col-0). Homozygous seeds of *atmrp1-1* mutant and wild-type WS plants and of homozygous *atmrp1-2* mutant and wild-type Col-0 plants were surface-sterilized with 0.05%(w/v) sodium hypochlorite/ 0.1%(w/v) Tween20, washed exhaustively with sterile water, and germinated for 48h at 4°C on solid MS medium (pH 5.7) containing 1% (w/v) sucrose. Thereafter, the plates were grown vertically under controlled environmental conditions (24 ± 2°C; continuous cool fluorescent illumination of 75µmol m^{-2} s^{-1} light intensity; 70% relative humidity) for 12 days before measuring primary root length. For subsequent detailed screens of the sensitivity of growth to sodium arsenite at the whole plant level, surface-sterilized mutant and wild-type seeds were germinated for 48h at 4°C on solid MS medium containing 0.5g/liter MES, 0.9% (w/v) Difco-Bacto agar, 1% (w/v) sucrose, and the indicated concentrations of sodium arsenite in plant tissue culture vessels before transfer to a plant growth room for growth for a further 14 days. In all of the phenotypic screens, precautions were taken to ensure that several independent mutant and wild-type seed batches were treated and screened in parallel under identical conditions. To analyze arsenic tolerant germination, seeds were placed on medium with indicated concentrations of sodium arsenite and germination procedure was followed.

Phospho-status identification of NBD2 serine residues in plant proteins

Interaction of plant leaf extracts with NBD2 antibody and their serine phosphorylation status were investigated following the binding conditions described previously [12]. Binding with NBD2 antibody was done for 4h at 4°C and proteins were immunoprecipitated with Protein A sepharose beads. The reaction mixture was analyzed in 10% SDSPAGE [13] and western blotted with phospho-serine antibody (Qiagen) following manufacturer instructions. All protein concentrations were determined using Bradford method [14].

STATISTICAL ANALYSIS

All statistical analysis was done from graph of root length in presence of sodium arsenite. P value ($^{*}P<=0.05$) Two sided was obtained by Ky plot software. By conventional criteria these are considered to be statistically significant.

RESULTS AND DISCUSSION

Plant growth in presence of arsenite salt

Screens were initiated to assess the sensitivity of *atmrp1-1* and *atmrp1-2* T-DNA homozygous mutants by comparison with wild type plants to the stress effects of increasing concentrations of arsenite salt. Seeds of *atmrp1-1* and *atmrp1-2* homozygous plants and their corresponding wild type WS and Col-0 were germinated and grown in the 16/8 h photoperiod on sterile plates and plant tissue culture vessels containing 0-100µM sodium arsenite and root growth with plant growths were observed. Neither of these T-DNA insertion mutants are more susceptible than their wild type counterparts with increasing arsenite salt concentrations (0-100µM) and root length measurements show greater growth retardation of these *atmrp1-1* and *atmrp1-2* mutants (Figure 1A). Root length measurement tests were done for 3 times.

Comparison of plant growth of *atmrp1-1* mutant with corresponding wild type WS plants in MS plates containing arsenite salt of 0-100µM are shown in Figure 1B. This shows the diminished *atmrp1-1* plant growth in presence of arsenite. Growth retardation of *atmrp1-1* and *atmrp1-2* mutants in presence of arsenite salt concentrations 0-70µM were shown in bar diagrams (Figure 1C). Growth retardation measurement tests were done for 3 times.

Plant growth were hampered in *atmrp1-1* and *atmrp1-2* mutant plants compared to their corresponding wild types when they were treated with 50µM sodium arsenite mixed water for 3 weeks constantly in soil which indicates the extent of toxicity of arsenite salt and stress imposed on plant growth (Figure 1D). All these data show mutant plant growths were severely hampered by arsenite treatment which is the most important phenotype.

Immunoprecipitation and western blot of plant extracts

Pull down assay with NBD2 antibody [6] and western blot with phospho-serine antibody in *Arabidopsis thaliana* wild type and T-DNA mutant plant leaf extracts show 170kD MRP band in absence of arsenite in wild type WS and Col-0 plants (Figure 2; lane a and lane c) which is absent in mutants *atmrp1-1* and *atmrp1-2* (Figure 2; lane b and lane d). Equal amounts of wild type and mutant proteins from each strain were loaded in gel.

Results indicate the wild type Col and WS NBD2 serine

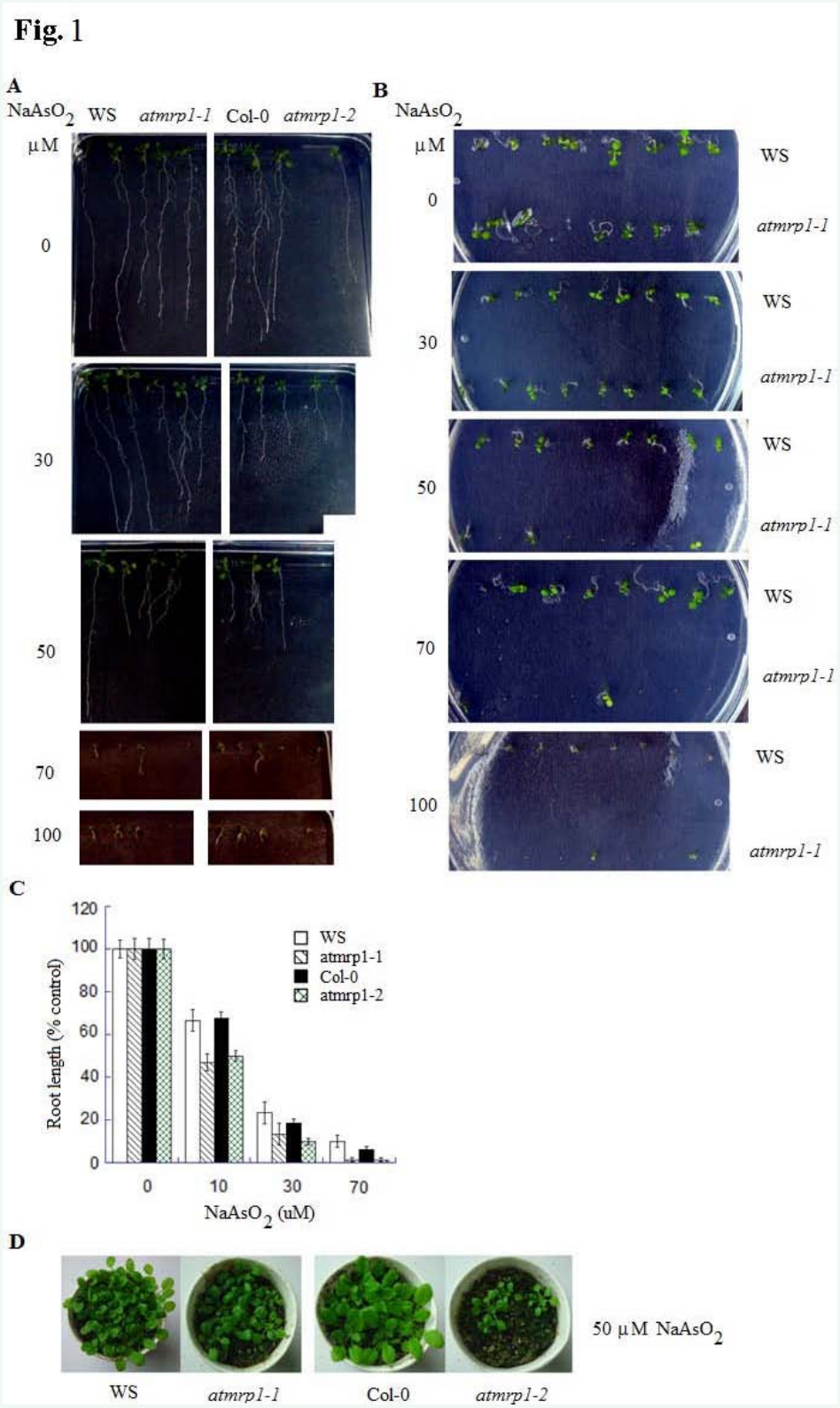

Figure 1 Differential sensitivities of growth of wild type and mutant plants in presence of sodium arsenite: Difference in root growth of wild type WS, Col-0, and mutant *atmrp1-1, atmrp1-2* plants in MS plate containing increasing sodium arsenite (0-100µM). B. Difference in plant growth of WS and *atmrp1-1* in MS plate in presence of sodium arsenite (0-100µM). C. Comparison of root lengths (% control) in presence of sodium arsenite (0-70µM) (from three independent experiments with four replicates each). P-values obtained by Ky plot software for arsenite equals * (P<=0.05) Two sided. By conventional criteria these are considered to be statistically significant. D. Wild type and mutant plant growth after germination in soil watered with 50µM sodium arsenite for 3 weeks. One representative plant out of four was photographed 21 d after arsenite treatment.

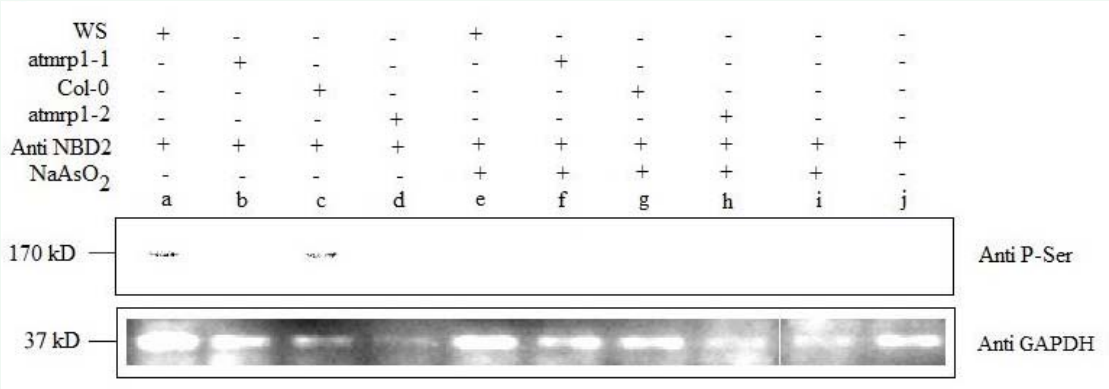

Figure 2 Pull down assay to identify phospho status of serine using anti p-Ser antibody: *Arabidopsis thaliana* wild type and T-DNA mutant plants grown in absence (a-d) and presence (e-i) of sodium arsenite (50μM) and proteins were pull down with anti-NBD2 antibody and western blot with phospho serine antibody. Anti-GAPDH antibody was used for loading control. Some of the data were acquired from separate gels. The position of AtMRP1 (170kD) and GAPDH (37kD) are indicated with black arrowhead.
Abbreviations: ABC: ATP Binding Cassette; **MRP:** Multidrug Resistance Protein; **NBD2:** Nucleotide Binding Domain 2; **GSH:** Glutathione; **MSD:** Membrane spanning domain

triad in absence of arsenite remain in phosphorylated state but in presence of arsenite they are in dephosphorylated state. Wild type MRP1 protein can transport arsenite by serine triad phosphorylation which are absent in *atmrp1-1* and *atmrp1-2* mutants. Earlier results showed with *in vitro E. coli* expressed NBD2 proteins, serine triad phosphorylation happened [6] in presence of arsenite. But in plant this phosphorylation is absent in MRP1 mutants which indicate different arsenite stress signaling operate in transport mechanism of plant proteins.

CONCLUSION

Results present here clearly show the growth retardation of atmrp1-1 and atmrp1-2 mutant plants in presence of increasing amount of arsenite salt. Earlier work with arsenite salts showed change in serine phosphorylation in different mutants of in vitro expressed NBD2 domain serine triad. Here the effect of arsenite was again shown in severe plant growth retardation of MRP1 T-DNA homozygous mutants which indicates the stress effects on *Arabidopsis thaliana* MRP1. As the proper functioning of MRP1 mutants are disturbed they fail to sequester arsenite in vacuoles and the plant growth was severely hampered. Phosphorylation in plant NBD2 serine residues were checked by immunoprecipitation with NBD2 antibody followed by western blot with phospho-serine antibody. It seems different potential signaling event in arsenite transport mechanism operate in plant proteins. In arsenite treated plants serine dephosphorylation occurred which indicate in plants presence of arsenite stress initiate some different transport mechanism.

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